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(57) Abstract

A new class of phosphodiesteric oligonucleotides which exert a selective cytotoxic activity on tumoural cells, having the nucleotide sequence of formula (I): $N\text{-}T_x\text{-}(G_aT_{a'})_{a''}\text{-}(G_bT_{b'})_{b''}\text{-}(G_cT_{c'})_{c''}\text{-}(G_dT_{d'})_{d''}\text{-}(G_eT_{e'})_{e''}\text{-}(G_fT_{f'})_{f''}\text{-}(G_gT_{g'})_{g''}\text{-}N'$ with orientation 5'-3' or 3'-5', where N and N', equal or different from each other, are T or G; x ranges from 0 to 8; a, b, c, d, e, f and g, equal or different from each other, range from 0 to 10; a', b', c', d', e', f' and g', equal or different from each other, range from 0 to 30; and a'', b'', c'', d'', e'', f'' and g'', equal or different from each other, range from 0 to 16. Furthermore, pharmaceutical compositions containing at least one of said phosphodiesteric oligonucleotides and their therapeutic use as antitumoural agents.

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A CLASS OF OLIGONUCLEOTIDES, THERAPEUTICALLY USEFUL AS ANTITUMOURAL AGENTS

FIELD OF THE INVENTION

The present invention relates to a new class of phosphodiesteric oligonucleotides, which exert a selective cytotoxic activity on tumoural cells.

STATE OF THE ART

One of the main targets of the research on cancer is the identification of drugs able to act in a selective way on tumoural cells, without exerting harmful effects on the healthy ones. Antitumoural drugs presently used in clinical trials do not discriminate neoplastic cells from the healthy ones, since their target is generally DNA replication or the interference with metabolites. The difference of toxicity which is observed in tumoural cells and which permits the clinical use of said antitumoural drugs is due to the fact that the transformed cells replicate and metabolize more rapidly than the healthy ones. The consequences of the lack of specific tumoural targets in the mechanism of action of traditional chemotherapeutic agents are unavoidable side effects at the systemic level.

In the last few years, oligonucleotides have been studied as antitumoural agents. As a matter of fact, with respect to traditional drugs, nucleic acids, which can be effectively taken up by cells via either a receptor-mediated endocytosis mechanism and/or pinocytosis, exhibit higher possibilities of selectively acting on specific targets, such as the products of oncogenes or of drug-resistance genes, since their action is based on the specific sequence of bases with which the genetic information is codified.

However, the use of nucleotide sequences in human therapy is strongly limited by the short half-life period of natural oligonucleotides in the serum and in the cells, which is due to the presence of RNases. This limitation has been overcome by using oligonucleotides where the internucleoside phosphatidic chain is modified, for example obtaining phosphotriesters, phosphonates and phosphorothioates, which are more resistant to the attack of nucleases; moreover, in order to favour the penetration of oligonucleotides into cells, on whose mechanism several hypotheses have been put forward, oligonucleotides have been advantageously linked to poly-L-lysine chains or to cholesterol residues.

Recent experiments on rats have shown that oligonucleotides, when intravenously, intraperitoneally or differently administered, can achieve pharmacologically active concentrations in the target organs and are very well tolerated at the systemic level (Vlassov V.V. et al., *FEBS LETTERS*, **327**: 271-274; 1991).

The above oligonucleotide sequences can act according to several mechanisms of action and cellular targets (Hèléne C. and Toulmé J.J., *Biochimica et Biophysica Acta*, 1049: 99-125; 1990).

The approach which is currently more studied in the treatment of tumours consists in the use of antisense oligonucleotides; in this case, the stop of the translation of specific mRNAs is performed by mimicking the natural process of half-life regulation of the mRNA present in cells. More precisely, an oligonucleotide which is complementary to a specific sequence of mRNA forms DNA-RNA partial hybrids which lead to the stop of the translation of the message and/or to their degradation.

However, the application of this strategy gives rise to remarkable difficulties and disadvantages which are essentially due to the low extra- and intra-cellular half-life period of oligonucleotides with respect to the rapid turnover of mRNAs.

Another strategy, basing its mode of action at an earlier step with respect to the antisense inhibition, consists in the formation of the intermolecular DNA triple helix. This kind of approach is less studied than the previous one but it offers the potentiality of performing a block directly at the transcription level. Even in this case, some applicability limitations are noticeable, mainly due to the need of identifying suitable regions of the gene where homopurinic/homopyridinic sequences are present.

More specifically, according to the above mechanism of action, the Applicant has found that the oligonucleotide having the sequence 5'-TGTGTTTTTGTTTTGTTGGTTTTGTTT-3', is able to inhibit the mRNA transcription of the mdr1 gene, in the MDR tumoural cell line, which codifies a transmembrane glycoprotein responsible for drug-resistance (Scaggiante B. et al., *FEBS Letters*, **352**:380-384; 1994).

Finally, oligonucleotides can be used also as a target of specific proteins. It is known that interactions between single-stranded DNA and/or RNA and proteins are at the basis of essential regulation mechanisms of the replication, transcription, repair and recombination of DNA, or of the ripening and translation of mRNAs. These proteins are present in all living organisms, from prokariotes to eukariotes, and they are known as "single-strand DNA-binding proteins" (SSBs). Often, the SSBs do not recognise specific sequences, but they recognize specific motifs. (Holligsworth M.A. et al., *Nucleic Acids Research*, **22**: 1138-1146; 1994).

A. Aharoni et al. (*Nucleic Acids Research*, 1993, Vol. 21, N° 22, p. 5521-5528) illustrate the ability of different DNA segments, among which the $d(GT)_{10}$ sequence, to bind a new protein called PGB which has been identified in human fibroblasts; however, no biological significance of the affinity of the above sequence for this protein is suggested.

So far, in the state of the art, no oligonucleotide sequences have been proposed which can bind proteins with a specific and selective cytotoxic effect on neoplastic cells.

SUMMARY OF THE INVENTION

The Applicant has now found a new class of phosphodiesteric oligonucleotides having a sequence of formula (I):

$$N\text{-}T_x\text{-}(G_aT_{a'})_{a''}\text{-}(G_bT_{b'})_{b''}\text{-}(G_cT_{c'})_{c''}\text{-}(G_dT_{d'})_{d''}\text{-}(G_eT_{e'})_{e''}\text{-}(G_fT_{f'})_{f''}\text{-}(G_gT_{g'})_{g''}\text{-}N' \quad (I)$$

with orientation 5'-3' or 3'-5', where N and N', equal or different from each other, are T or G; x ranges from 0 to 8; a, b, c, d, e, f and g, equal or different from each other, range from 0 to 10; a', b', c', d', e', f' and g', equal or different from each other, range from 0 to 30; and a'', b'', c'', d'', e'', f'' and g'', equal or different from each other, range from 0 to 16,

with the exception of the sequences 5'-TGTGTTTTTGTTTTGTTGGTTTTGTTT-3' (SEQ ID N°:1) and 5'-GTGTGTGTGTGTGTGTGTGT-3' (SEQ ID N° :11).

Surprisingly, said phosphodiesteric oligonucleotides are able to exert a selective cytotoxic activity on tumoural cells.

Further objects of the present invention are pharmaceutical compositions containing at least one of said phosphodiesteric oligonucleotides and their use in the treatment of tumours.

DETAILED DESCRIPTION OF THE INVENTION

The features and the advantages of the new class of phosphodiesteric oligonucleotides, of their therapeutic use as antitumoural agents and of the pharmaceutical compositions containing them, according to the present invention, will be better described in the following detailed description.

The above sequences of oligodeoxyribonucleotides of formula (I) are able to act as specific and selective antitumoural agents. In these oligonucleotidic sequences, N and N' can be T or G. Moreover, said sequences:

- contain a total number of nucleotides ranging from 10 to 60, preferably from 20 to 40;
- contain a number of T nucleotides ranging from 10 to 40, preferably from 16 to 32;
- contain a number of G nucleotides ranging from 1 to 25, preferably from 2 to 10.

Among the oligonucleotides of formula (I), the sequence 5'-TGTGTTTTTGTTTTGTTGGTTTTGTTT-3' (SEQ ID N°:1), wherein N=N'=T, x=0, a=b=c=d=f=1, e=2, a'=1, b'=5, c'=4, d'=2, e'=4, f'=2 and a''=b''=c''=d''=e''=f''=1, the remaining variables being 0, is already known in the state of the art. Nevertheless, it has been described only the ability of said oligonucleotide to inhibit the production of a glycoprotein responsible for the drug-resistance in the MDR tumoural cell line, by inhibiting the transcription of the corresponding mRNA, with the mechanism of the molecular DNA triple-helix (Scaggiante B. et al.; reference cited above.).

Also the sequence 5'-GTGTGTGTGTGTGTGTGTGT-3' (SEQ ID N°:11) corresponding to the formula (I) wherein N=G, N'=T, x=1, a=a'=1, a''=8, b=b''=1 and b'=0, the remaining variables being 0, is already known in the state of the art, but no specific biological activity is reported (A. Aharoni et al.; reference cited above).

Among the phosphodiesteric oligonucleotides of the invention, are particularly active the ones having the following sequences:

5'-TGTTGTTGTTGTTGTTGTTGTTGTTGT-3' (SEQ ID N°:2), corresponding to the formula (I), wherein N=N'=T, x=0, a=1, a'=2, a''=8, b=b''=1 and b'=0, the remaining variables being 0;

5'-TGTTTGTTTGTTTGTTTGTTTGTTTGT-3' (SEQ ID N°:3), corresponding to the formula (I), wherein N=N'=T, x=0, a=1, a'=3, a''=6, b=b''=1 and b'=0, the remaining variables being 0;

5'-TGTTTTGTTTTGTTTTGTTTTGTTTTGT-3' (SEQ ID N°:4), corresponding to the formula (I), wherein N=N'=T, x=0, a=1, a'=4, a''=5, b=b''=1 and b'=0, the remaining variables being 0;

5'-TGTTTTTGTTTTTGTTTTTGTTTTTGT-3' (SEQ ID N°:5), corresponding to the formula (I), wherein N=N'=T, x=0, a=1, a'=5, a''=4, b=b''=1 and b'=0, the remaining variables being 0;

5'-TTTGTTTTTTGTTTTTTGTTTTTTGTTTTTTGTTT-3' (SEQ ID N°:6), corresponding to the formula (I), wherein N=N'=T, x=2, a=b=1, a'=6, a''=4, b'=2 and b''=1, the remaining variables being 0;

5'-TGTTTTTTTGTTTTTTTGTTTTTTTGTTTTTTTGT-3' (SEQ ID N°:7), corresponding to the formula (I), wherein N=N'=T, x=0, a=b=1, a'=7, a''=4, b'=0 and b''=1, the remaining variables being 0;

5'-GTTTGTTTGTTTGTTTGTTTGTTTGTG-3' (SEQ ID N°:8), corresponding to the formula (I), wherein N=N'=G, x=3, a=b=1, a'=3, a''=5, b'=b''=1, the remaining variables being 0;

5'-TTTGTTGTTTTTGTTTTGTTTT-3' (SEQ ID N°:9), corresponding to the formula (I), wherein N=N'=T, x=2, a=b=c=d=1, a'=2, b'=5, c'=4, d'=3 and a''=b''=c''=d''=1, the remaining variables being 0;

5'-TTTTTTTTGTTTTTTTTGTTTTTTTT-3' (SEQ ID N°:10), corresponding to the formula (I), wherein N=N'=T, x=7, a=b=1, a'=8, b'=7, and a''=b''=1, the remaining variables being 0;

5'-GGTTTGTTTGTTTGTTTGTTTGTTTGG-3' (SEQ ID N°:12), corresponding to the formula (I), wherein N=N'=G, a=1, a'=3, a''=6 and b=b''=1, the remaining variables being 0;

5'-TGTTTGTTTGTTTGTTTGTTTGTTTGG-3' (SEQ ID N°:13), corresponding to the formula (I), wherein N=T, N'=G, a=1, a'=3, a''=6 and b=b''=1, the remaining variables being 0;

5'-TGGTTGGTTGGTTGGTTGGTTGGTTGGT-3' (SEQ ID N°:14), corresponding to the formula (I), wherein N=N'=T, a=2, a'=2, a''=6, b=2 and b''=1, the remaining variables being 0;

5'-TTTTTGTTTTTGTTTTTGTTTTTGTTTTTGTTTTT-3' (SEQ ID N°:15), corresponding to the formula (I), wherein N=N'=T, a=b=b''=1, a'=5, a''=b'=4 and x=4, the remaining variables being 0;

5'-TTTGTTTTGGTTGTTTTGTTT-3' (SEQ ID N°:16), corresponding to the formula (I), wherein N=N'=T, x=2, a=c=d=1, b=2, a'=c'=4, b'=d'=2 and a''=b''=c''=d''=1, the remaining variables being 0;

5'-TGTTTGTTTGTTTGTTTGT-3' (SEQ ID N°:17), corresponding to the formula (I), wherein N=N'=T, a=b= b''=1 and a'=a''=3, the remaining variables being 0;

5'-TGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGT-3' (SEQ ID N°:18), corresponding to the formula (I), wherein N=N'=T, a=1, a'=3, a''=12 and b=b''=1, the remaining variables being 0;

5'-TGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGT-3' (SEQ ID N°:19), corresponding to the formula (I), wherein N=N'=T, a=1, a'=30, a''=1 and b=b''=1, the remaining variables being 0.

Moreover, the oligonucleotides of the present invention can be modified on the internucleosidic phosphatidic groups, on the terminal phosphate groups, on the bases and on the sugars, according to methods known in the state of the art, with the aim of increasing their resistance to the attack of the extra- and intra-cellular nucleases. In particular, the oligonucleotide sequences of the invention can be chemically modified on the terminal and/or internucleosidic phosphate groups to give methylphosphonates, phosphoroamidates, phosphorothioates, phosphorodithioates and phosphoroselenates. Among the 3' and/or 5' phosphoroamidate analogs, are preferred the derivatizations with methoxyethylamine, dodecylamine and octadecylamine. Furthermore, said sequences can be derivatized on the sugar mojeties to give L-desoxyribose analogs, 2'-O-allyl- and 2'-O-methyl-desoxyribose derivatives.

For illustative but not limitative purposes, the following examples are reported.

EXAMPLE 1

Preparation of oligonucleotides of formula (I), according to the present invention.

The phosphodiesteric oligonucleotides according to the present invention have been synthesized by means of a DNA automated synthesizer by Applied Biosystem, model 380 B, by using the phosphoroamidite method, according to a 1 µM standard procedure. The oligonucleotides thus obtained were then deprotected by heating at 56°C overnight.

The oligonucletoides were then purified by FPLC on a MONO Q HR 5/5 column, by using an ammoniun bicarbonate gradient. The purified oligonucleotides were freeze-dried and then suspended in 300 µl of NaCl (0.9%w). Their concentration was spectrophotochemically determined at the wavelength of 260 nm, at the temperature of 60°C.

The purity of the thus obtained oligonucleotides, mesured by electrophoresis on a 15% polyacrylamide gel in 0.1M acetic acid/7M urea, under denaturating conditions, turned out to be in the range of 80 to 90%. The yield ranged from 30 to 60%.

Finally, the oligonucleotides were sterilized by filtration on membranes with a porosity of 0.2 µm.

EXAMPLE 2

Preparation of phosphorothioate derivatives of the oligonucleotides of formula (I), according to the present invention.

Fully phosphorothioate modified oligonucleotides and 3'-phosphorothioate modified oligonucleotides were synthesized by using the phosphoroamidite method, according to a 1 µM standard procedure, as described in Example 1. The derivatized oligonucleotides were then purified by gel permeation chromatography on Sephadex G50 fine resin, using 0.05M ammoniun bicarbonate. The eluted fractions were monitored by UV absorbance, at 254 nm.The purified derivatized oligonucleotides were freeze-dried and then suspended in 300 µl of 0.9% NaCl. Their concentration was spectrophotochemically determined by UV absorbance at the wavelength of 260 nm, at the temperature of 60°C.

The purity of the thus obtained derivatized oligonucleotides was mesured by gel electrophoresis, under denaturating conditions, as described in Example 1. The purity of the samples turned out to be of about 80%. The reaction yield ranged from 50 to 60%.

Finally, the derivatized oligonucleotides were sterilized by filtration on membranes with a porosity of 0.2 µm.

BIOLOGICAL ACTIVITY

The oligonucleotide sequences according to the present invention exhibited the ability to exert, even only after one single administration, a significant and specific cytotoxic activity in human tumoural lines. Moreover, the selectivity of the cytotoxic action of siad sequences for tumoural cells was proved by the lack of effects on human healthy cells.

In particular, toxicity tests were performed on several human cellular lines.

The following lines were used:

- CCRF-CEM lymphoblastic line, for a liquid tumour model;
- epithelial line of LoVo 109 colon adenocarcinoma, for a solid tumour model;
- U937 monocyte line, for a solid tumour line, in particular lymphoma.

The results of the different experimental models showed that the oligonucleotidic sequences according to the present invention are very active both on liquid (lymphoblastic) tumours and on solid (lymphoid) tumours, and exhibit an effective action on epithelial solid tumours.

EXAMPLE 3

Evaluation of the cytotoxicity of the oligonucleotides according to the present invention on CCRF-CEM tumoural cells.

CCRF-CEM tumoural cells were cultured in RPMI 1640 medium containing 10%w fetal calf serum, 20 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Gln.

The cells were then seeded at the density of $5x10^4$ cells/ml in a 96 wells microtiter (100 µl cell suspension, equal to $5x10^3$ total cells for each well).

After 24 hours of incubation, the oligonucleotides having sequences SEQ ID N°:1 to SEQ ID N°:10, SEQ ID N°:12 to SEQ ID N°:16, SEQ ID N°:18 and SEQ ID

N°:19, according to the present invention, were added directly to the culture medium in concentrations ranging from 2.5 to 30 μM.

The effect of the administration of the above oligonucleotides on the cellular growth and viability was evaluated after 24, 48 and 72 hours, by incorporation of 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), which was added at the concentration of 0.5 mg/ml.

After 4 hours of incubation in the presence of the dye, the cells were centrifuged for 8 minutes at 400xg. The medium was then removed by suction; the cells were disrupted and the dye was solubilized in 200 μl DMSO.

The absorbance was read spectrophotometrically, at the weavelengths of 540 and 690 nm.

The percentage of cellular growth was measured assuming as 100% the cellular growth of untreated cells.

The thus obtained results are reported in Table 1.

Table 1

Cytotoxic effect of the oligonucleotides according to the present invention on CCRF-CEM cells, after 72 hours from the administration of the sequences.

Oligonucleotide	% Cellular growth reduction with oligonucleotide concentrations of		
	5μM	7.5μM	15μM
SEQ ID N°: 1	62±17	79±13	92±6
SEQ ID N°: 2	42±10	62±6	79±4
SEQ ID N°: 3	59±19	72±11	85±7
SEQ ID N°: 4	43±12	57±7	78±2
SEQ ID N°: 5	46±7	65±5	79±3
SEQ ID N°: 6	50±14	66±11	87±4
SEQ ID N°: 7	58±14	75±6	83±4
SEQ ID N°: 8	51±12	67±6	83±5
SEQ ID N°: 9	53±14	67±9	79±5
SEQ ID N°:10	36±17	48±15	76±11
SEQ ID N°:12	46±13	66±12	82±8
SEQ ID N°:13	41±12	66±11	83±8
SEQ ID N°:14	36±18	47±17	70±15
SEQ ID N°:15	59±12	67±13	87±9
SEQ ID N°:16	36±5	48±10	71±11
SEQ ID N°:18	55±20	69±11	93±3
SEQ ID N°:19	34±22	46±21	71±10

The above data outline a significant reduction of the cellular growth, which is detectable 48 hours after the administration of the oligonucleotide of the invention.

Moreover, the cytotoxic effects of the sequence SEQ ID N°:3 were evaluated for concentrations of 7.5 μM, on CCRF-CEM cells, after 24, 48 or 72 hours from the

administration of the sequence. A decrease of 5% in cellular growth was detected after 24 hours and a decrease of 31% was noted after 48 hours.

EXAMPLE 4

Evaluation of the relevance of the repeating unit (GT) in the specificity of the cytotoxic action of the oligonucleotides of the present invention.

In order to check the relevance of the repeating unit (GT_n) in the sequences according to the present invention, was calculated the cytotoxic activity of oligonucleotidic sequences containing (CT_n), (AT_n), (GC_n) and (GA_n) as repeating unit, and more specifically of the following oligonucleotide sequences:

5'-TCTTTCTTTCTTTCTTTCTTTCTTTCT-3', which will be indicated as (CT);

5'-TATTTATTTATTTATTTATTTATTTAT-3', which will be indicated as (AT);

5'-CGCCCGCCCGCCCGCCCGCCCGCCCGC-3', which will be indicated as (GC); and

5'-AGAAAGAAAGAAAGAAAGAAAGAAAGA-3', which will be indicated as (GA).

These sequences, which were synthesized and purified as described in Example 1, were administered to CCRF-CEM cells at concentrations of 5, 7.5 and 15μM, according to the procedure described in Example 3. The results, detected after 72 hours from the administration of the sequences, are reported in Table 2.

Table 2

Cytotoxic effect of oligonucleotides with different repeating units on the growth of CCRF-CEM cells, after 72 hours from the administration of the sequences.

Oligonucleotide	% Cellular growth reduction with oligonucleotide concentrations of		
	5μM	7.5μM	15μM
(CT)	4±10	9±9	11±11
(AT)	13±11	17±11	25±8
(GC)	12±11	23±13	28±19
(GA)	20±11	26±6	52±10

The results reported above prove that the oligonucleotides having (CT), (AT) and (GC) repeating units cannot significantly alter the cellular growth, while the (GA) oligonucleotide is poorly toxic only if used at high concentrations (15μM), showing a cellular growth inhibition of 52%.

EXAMPLE 5

Evaluation of the relevance of the features of formula (I) to the cytotoxic activity exerted by the oligonucleotides of the present invention.

In order to check the relevance of the specific features of the sequences of formula (I) according to the present invention, was evaluated the cytotoxic activity of oligonucleotidic sequences containing the repeating unit (GT_n), but having the following characteristics with respect to formula (I):

A) N and/or N' are different from G and T.

More specifically, the following oligonucleotide sequences were tested:

5'-AGTTTGTTTGTTTGTTTGTTTGTTTGA-3', which will be indicated as SEQ A1;

5'-CGTTTGTTTGTTTGTTTGTTTGTTTGC-3', which will be indicated as SEQ A2;

5'-TGTTTGTTTGTTTGTTTGTTTGTTTGC-3', which will be indicated as SEQ A3.

B) The sequence has flanking fragments containing C and T bases.

More specifically, the following oligonucleotide sequences were tested:

5'-CTTTTCTTTCTTTGTGTGTGTGTTTCTTTCTTTTC-3', which will be indicated as SEQ B1;

5'-TTTCTTTCTTTGTTGTTGTTGTTGTTTCTTTCT-3', which will be indicated as SEQ B2;

5'-TCTTTCTTTGTTTGTTTGTTTGTTTGTTTCTTTCT-3', which will be indicated as SEQ B3;

5'-TTTCTTTGTTTTGTTTTGTTTTGTTTTGTTTTCTTT-3', which will be indicated as SEQ B4;

5'-TCTTTGTTTTTGTTTTTGTTTTTGTTTTTGTTTTTGTTTCT-3', which will be indicated as SEQ B5.

C) The sequence has a number of nucleotides lower than 10.

More specifically, the following oligonucleotide sequence was tested:

5'-TGTTTGT-3', which will be indicated as SEQ C1.

D) The oligonucleotide has at least a long stretch of sequence containing only G bases. More specifically, the following oligonucleotide sequence was tested: 5'-TTGGTTGTTTTTGGGGGGGGGGGGTGG-3', which will be indicated as SEQ D1.

These sequences, which were synthesized and purified as described in Example 1, were administered to CCRF-CEM cells at concentrations of 5, 7.5 and 15μM, according to the procedure described in Example 3. The results, detected after 72 hours from the administration of the sequences, are reported in Table 3.

Table 3

Cytotoxic effect of oligonucleotides with different sequence features on the growth of CCRF-CEM cells, after 72 hours from the administration of the same oligonucleotides.

Oligonucleotide	% Cellular growth reduction with oligonucleotide concentrations of		
	5μM	7.5μM	15μM
SEQ A1	12±11	17±10	23±15
SEQ A2	5±10	15±9	24±20
SEQ A3	11±10	14±6	23±11
SEQ B1	9±10	7±8	9±9
SEQ B2	9±9	11±9	26±18
SEQ B3	8±8	13±10	19±13
SEQ B4	15±4	22±8	26±16
SEQ B5	13±11	23±8	26±19
SEQ C1	2±5	7±5	15±14
SEQ D1	6±8	9±9	17±6

The results reported above demonstrate that:

A) when N and/or N' in the sequences of formula (I) are other than G and T, the oligonucleotides do not show any significant cytotoxic effects;

B) oligonucleotides having flanking sequences containing C and/or T bases do not exert significant cytotoxic activities;

C) sequences having a number of nucleotides lower than 10 are not toxic with respect to cellular growth;

D) sequences with a number of adjacent G bases of 11 or more do not inhibit cellular growth.

EXAMPLE 6

Selectivity of the cytotoxic activity of the oligonucleotides of the present invention.

Experimental trials were performed in order to evaluate the selectivity of the cytotoxic activity of the oligonucleotides of formula (I) and the lack of cytotoxic effects on healthy human cells.

The following cultures were used:

- primary cultures of lymphocytes obtained from peripheral blood, seeded at $5x10^5$ cells/ml ($5x10^4$ cells/well); these lymphocytes were used both during the resting phase and after activation with 10 µg/ml of lectin, 24 hours before the addition of the oligonucleotides;
- primary cultures of fibroblasts from human skin.

The above cells were treated with the phosphodiesteric oligonucleotide SEQ ID N°:3 of the invention and with the (CT) sequence described above, according to the procedure described in Example 3.

The obtained results are reported in Table 4.

Table 4.

Lack of cytotoxic effects of the oligonucleotides according to the present invention in cultures of healthy human cells, 72 hours after the administration of the sequences.

Oligonucleotide	(%) Cellular growth reduction with oligonucleotide concentrations of			
	7.5µM	15µM	30µM	50µM
Activated Lymphocytes				
SEQ ID N°:3	n.d.	17±9	8±12	15±13
(CT) Sequence	n.d.	12±7	2±6	7±9
Resting Lymphocytes				
SEQ ID N°:3	n.d.	9±3	13±13	20±5
(CT) Sequence	n.d.	2±2	4±5	4±5
Fibroblasts				
SEQ ID N°:3	2±9	5±6	3±9	n.d.
(CT) Sequence	3±7	14±7	10±7	n.d.

The results reported above show that the sequences of the invention have no cytotoxic effects on the growth and viability of primary cultures of healthy cells, thus indicating a highly selective activity of said oligonucleotides toward tumoural cells.

EXAMPLE 7

Evaluation of the cytotoxicity of the oligonucleotides according to the present invention in drug-sensitive and drug-resistant human tumoural lines.

At the light of the enormous importance that drug-resistance plays in the treatment of many human tumourals at a primary onset or after partial remission, experimental tests of cytotoxicity were performed on the following tumoural lines:

- CEM-VLB100 drug-resistant lymphoblastic line;
- drug-sensitive epithelial cells from LoVo 109 colon adenocarcinoma;
- drug-resistant epithelial cells from LoVo Dx colon adenocarcinoma;
- monocytes cell lines from the U937 lymphoma.

These cells were treated with the phosphodiesteric oligonucleotides SEQ ID N°:1 to SEQ ID N°:7, SEQ ID N°:9 and SEQ ID N°:12 to SEQ ID N°:14 of the present invention, according to the procedure described in Example 3.

For comparative purposes, the cells were also treated, under the same operating conditions, with the oligonucleotides (CT), as described in Example 4, SEQ A1, SEQ A3 and SEQ D1, as described in Example 5.

Again for comparative purposes, the above tumoural cellular lines were also treated, under the same operating conditions, with the 3'-phosphorothioate derivative of SEQ ID N°:3, prepared as described in Example 2 (hereinafter referred to as SEQ ID N°:3-3'-phosphorothioate).

The obtained results are reported in Table 5.

Table 5.

Cytotoxicity of oligonucleotides according to the present invention and of modified oligonucleotides in drug-sensitive and drug-resistant human tumoural lines, after 72 hours from the administration of the sequences.

Oligonucleotide	(%) Cellular growth reduction with oligonucleotide concentrations of			
	5μM	7.5μM	15μM	30μM
CEM-VLB100				
SEQ ID N°:1	57±18	66±9	73±6	n.d.
SEQ ID N°:3	55±14	69±5	78±10	n.d.
SEQ ID N°3-3'-phosphor.	52±14	n.d.	n.d.	n.d.
SEQ ID N°:9	30±7	41±6	60±8	n.d.
(CT)	9±4	20±6	37±12	n.d.
SEQ D1	0±9	4±12	6±12	n.d.
U937				
SEQ ID N°:2	58±16	66±11	86±7	n.d.
SEQ ID N°:3	50±11	63±7	74±8	n.d.
SEQ ID N°:4	45±18	66±12	78±15	n.d.
SEQ ID N°:5	44±21	59±21	76±12	n.d.
SEQ ID N°:6	53±21	65±19	76±112	n.d.
SEQ ID N°:7	45±21	59±19	76±13	n.d.
SEQ ID N°:12	59±9	69±3	79±5	n.d.
SEQ ID N°:13	49±16	64±14	80±9	n.d.
SEQ ID N°:14	67±16	73±10	86±6	n.d.
(CT)	0±15	1±19	1±13	n.d.
SEQ A3	8±7	21±5	34±8	n.d.
LoVo 109				
SEQ ID N°:3	n.d.	17±2	26±2	32±2
LoVo Dx				
SEQ ID N°:3	n.d.	17±9	27±9	44±3

The data reported above prove that the phosphodiesteric oligonucleotides according to the present invention are able to exert very significant cytotoxic effects also on drug-resistant tumoural lines, especially on those of lymphoblastic origin.

On the contrary, oligonucleotides with repeating units other than (GT_n), as well as with different sequence features with respect to the sequence of formula (I) do not significantly inhibit the cellular growth of drug-resistant tumoural lines.

Furthermore, the above data demonstrate that the cytotoxic activity of the 3'-phosphorothioate oligonucleotides is comparable to the one exerted by the corresponding unmodified phosphodiesteric oligonucleotides.

The oligonucleotides according to the present invention can be profitably used in the treatment of tumours both of liquid type, in particular of lymphoblastic origin, and of solid type, in particular lymphomas.

Said oligonucleotides are taken up by cells by receptor-mediated pynocytosis and endocytosis mechanisms. According to an hypothesis of mechanism non-limitative of the present invention, the phosphodiesteric oligonucleotides corresponding to formula (I) can selectively bind and sequester some proteins which are essential to the viability and growth of tumoural lines, and in particular some nuclear proteins which could be expressed only in transformed cells. In this case, said oligonucleotides, contrary to other cytoxic compounds, would specifically and selectively block proteins essential to tumoural proliferation, by meanwhile maintaining the viability of healthy cells.

Moreover, the oligonucleotide-protein interaction could protect the nucleic acid from the intracellular degradation, thus allowing the achievement of specific pharmacologic effects at doses lower than those used in the antisense or triple-helix systems.

Further objects of the present invention are pharmaceutical compositions containing as the active principle a therapeutically effective amount of at least a phosphodiesteric oligonucleotide having a sequence corresponding to formula (I).

Said compositions can be systemically administered both orally and parenterally, as well as topically and transdermally. Among the parenteral administrations, the intravenous, the intramuscular, the rectal and the intravaginal routes are preferred.

The therapeutically effective dose depends on the seriousness of the pathology, on the administration route and on the application conditions; furthermore, it depends on the the age, the weight and the general health state of the patient.

The compositions of the invention include all the formulations with pharmaceutically acceptable excipients, useful for the administration of the active compound in the form which is more suitable to the pathology and which can render the oligonucleotides of the invention remarkably bioavailable. Said formulations can advantageously comprise the oligonucleotides according to the present invention in association with carriers or ingredients able to increase their cellular uptake and to stabilize them to degradation.

In particular, injectable solutions or suspensions can be advantageously used, comprising said oligonucleotides in salted buffer, in physiological solution, in Ringer solution or in the solutions commonly used in the state of the art; said injectable solutions and suspensions are particularly suitable for general, endovenous, subcutaneous and intramuscular administrations.

Solid or semi-solid formulations are also suitable, in the form of inserts, gels or ointments for topical administration. In particular, the oligonucleotides of the invention can be advantageously prepared in the form of powder, tablet or freeze-dried solid, to be dissolved in a solution immediately before the parenteral use.

Liposomal formulations commonly used in the state of the art, both for parenteral and topical use, can also be particularly advantageous.

For oral administration, granules, tablets, pills and capsules are preferred; controlled-release formulations, known in the state of the art, are also suitable, such as micro- or nano-spheres based on lipids and/or polysaccharides.

For dermal or transdermal administration, creams, ointments or gels, where the active principle can be entrapped in slow-release microspheres, are preferred.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: SAICOM S.r.l.
(B) STREET: Padriciano, 99
(C) CITY: Trieste
(D) STATE: Trieste
(E) COUNTRY: ITALY
(F) POSTAL CODE (ZIP): 34012
(G) TELEPHONE: 040/3756611
(H) TELEFAX: 040/7797091

(ii) TITLE OF INVENTION: A new class of phosphodiesteric oligonucleotides, therapeutically useful as antitumoural agents.

(iii) NUMBER OF SEQUENCES: 19

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGTGTTTTTG TTTTGTTGGT TTTGTTT 27

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGTTGTTGTT GTTGTTGTTG TTGTTGT 27

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TGTTTGTTTG TTTGTTTGTT TGTTTGT 27

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGTTTTGTTT TGTTTTGTTT TGTTTTGT 28

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGTTTTTGTT TTTGTTTTTG TTTTTGT 27

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTTGTTTTTT GTTTTTTGTT TTTTGTTTTT TGTTT 35

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TGTTTTTTTG TTTTTTTGTT TTTTTGTTTT TTTGT 35

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTTTGTTTGT TTGTTTGTTT GTTTGTG 27

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTTGTTGTTT TTGTTTTGTT TT 22

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TTTTTTTTGT TTTTTTTGTT TTTTTT 26

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(D) OTHER INFORMATION: Cytotoxic oligonucleotide sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTGTGTGTGT GTGTGTGTGT 20

CLAIMS

1. A phosphodiesteric oligonucleotide having a sequence corresponding to formula (I)

$$N\text{-}T_x\text{-}(G_aT_{a'})_{a''}\text{-}(G_bT_{b'})_{b''}\text{-}(G_cT_{c'})_{c''}\text{-}(G_dT_{d'})_{d''}\text{-}(G_eT_{e'})_{e''}\text{-}(G_fT_{f'})_{f''}\text{-}(G_gT_{g'})_{g''}\text{-}N' \quad (I)$$

with orientation 5'-3' or 3'-5', where N and N', equal or different from each other, are T or G; x ranges from 0 to 8; a, b, c, d, e, f and g, equal or different from each other, range from 0 to 10; a', b', c', d', e', f' and g', equal or different from each other, range from 0 to 30; a'', b'', c'', d'', e'', f'' and g'', equal or different from each other, range from 0 to 16,

with the exclusion of the oligonucleotides having the sequences SEQ ID N°:1 and SEQ ID N°:11.

2. The oligonucleotide according to claim 2, characterized by having at least a derivatization on the internucleosidic phosphatidic groups, on the terminal phosphate groups, on the bases and/or on the sugars.

3. The oligonucleotide according to claim 1, characterized by the fact that said derivatization is selected from the group consisting of methylphosphonate, phosphoroamidate, phosphorothioate, phosphorodithioate, phosphoroselenate, L-desoxyribose, 2'-O-allyl- and 2'-O-methyl-desoxyribose.

4. The oligonucleotide according to claim 1 or 2, characterized by containing a number of nucleotides ranging from 10 to 60.

5. The oligonucleotide according to claim 4, characterized by the fact that said number of nucleotides ranges from 20 to 40.

6. The oligonucleotide according to claim 1 or 2, characterized by containing a number of T nucleotides ranging from 10 to 40.

7. The oligonucleotide according to claim 6, characterized by the fact that said number of T nucleotides ranges from 16 to 32.

8. The oligonucleotide according to claim 1 or 2, characterized by containing a number of G nucleotides ranging from 1 to 25.

9. The oligonucleotide according to claim 8, characterized by the fact that said number of G nucleotides ranges from 2 to 10.

10. The oligonucleotide according to claim 1, selected from the group consisting of the following sequences: SEQ ID N°:2, SEQ ID N°:3, SEQ ID N°:4, SEQ ID N°:5, SEQ ID N°:6; SEQ ID N°:7, SEQ ID N°:8, SEQ ID N°:9, SEQ ID N°:10, SEQ ID N°:12, SEQ ID N°:13, SEQ ID N°:14, SEQ ID N°:15, SEQ ID N°:16, SEQ ID N°:17, SEQ ID N°:18 and SEQ ID N°:19.

11.An oligonucleotide as described in anyone of claims 1 to 10, including the sequences SEQ ID N°:1 and SEQ ID N°:11, for use as a medicament.

12. The oligonucleotide according to claim 11, for treating tumours.

13. The oligonucleotide according to claim 12, characterized by the fact that said tumours are liquid tumours.

14. The oligonucleotide according to claim 12, characterized by the fact that said tumours are solid tumours.

15. The oligonucleotide according to claim 14, characterized by the fact that said solid tumours are lymphomas.

16. A pharmaceutical composition containing as the active principle a therapeutically effective amount of at least an oligonucleotide as described in anyone of claims 1 to 10, including the sequences SEQ ID N°:1 and SEQ ID N°:11, in combination with suitable excipients and/or diluents.

17. The pharmaceutical composition according to claim 16, characterized by being orally, parenterally, topically or transdermally administered.

18. The pharmaceutical composition according to claim 16, characterized by being in the form of injectable solution or suspension.

19. The pharmaceutical composition according to claim 16, characterized by being in the form of granules, tablets, pills, capsules, liposomes, freeze-dried solids, micro- or nano-spheres based on lipids and/or polysaccharides.

20. The pharmaceutical composition according to claim 16, characterized by being in the form of cream, ointment or gel.

INTERNATIONAL SEARCH REPORT

national Application No
PCT/EP 96/05388

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07H21/04 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MINERVA BIOTECNOLOGICA, (JUN 1995) VOL. 7, NO. 2, PP. 176-181., XP000671788 MORASSUTTI , C. ET AL.: "CORRELATION BETWEEN CYTOTOXIC EFFECT AND BINDING TO NUCLEAR PROTEINS OF OLIGOMERIC d(GT)n SEQUENCES IN HUMAN CANCER CCRF-CEM CELL-LINE" see the whole document ---	1,12
X	WO 94 07367 A (APOLLON, INC., USA;MAX-PLANCK-GESSELSHAFT ZUR FOREDERUNG DER WISSENSC) 14 April 1994 see page 12, line 29 - page 16, line 30 see page 22, line 1 - line 6 see page 25, line 5 - line 11	1-6,8,9, 11,16-20
Y	see claims --- -/--	11-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
15 May 1997	20. 05. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Andres, S

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INTERNATIONAL SEARCH REPORT

mational Application No
PCT/EP 96/05388

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, vol. 21, 1993, OXFORD GB, pages 5221-5228, XP002030696 AHARONI, A. ET AL.: "Characterization of a multisubunit human protein which selectively binds single stranded d(GA)n and d(GT)n sequence repeats in DNA " cited in the application	1,4,8
Y	see figure 5 ---	11-20
Y	FEBS LETTERS, vol. 352, 1994, AMSTERDAM NL, pages 380-384, XP002030697 SCAGGIANTE, B. ET AL.: "Effect of unmodified triple-helix-forming oligodeoxyribonucleotide targeted to human multidrug-resistance gene mdr1 in MDR cancer cells" cited in the application see the whole document ---	11-20
X	BIOCONJUGATE CHEM. (1994), 5(5), 390-9 , 1994, XP000465950 JONES, D. ET AL.: "Conjugates of Double-Stranded Oligonucleotides with Poly(ethylene glycol) and Keyhole Limpet Hemocyanin: A Model for Treating Systemic Lupus Erythematosus" see (TG)10 and (TG)25 ---	1,4-9
X	ANTI-CANCER DRUG DESIGN, vol. 6, December 1991, pages 609-646, XP000673329 CROOKE, R.M.: "In vitro toxicology and pharmacokinetics of antisense oligonucleotides" see page 614, line 15 - line 36 see page 628, last paragraph - page 630, line 38 see page 632, line 35 - line 48 see page 636, line 1 - line 16 see page 639, line 7 - line 13 ---	1-7
X	NUCLEIC ACIDS RESEARCH, vol. 21, no. 8, 25 April 1993, pages 1853-1856, XP002015215 ECKER D ET AL: "RATIONAL SCREENING OF OLIGONUCLEOTIDE COMBINATORIAL LIBRARIES FOR DRUG DISCOVERY" see table 2 ---	1-3,8,9, 11

1

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. CLIN. MICROBIOL. (1993), 31(4), 904-10, XP000673321 NIESTERS, H. ET AL.: "Rapid, polymerase chain reaction-based identification assays for Candida species" see Table 1, oligos 739 and 799	1,4-6,8, 9
X	NUCLEIC ACIDS RES. 21(16), 3911-12, 1993, XP002030698 KARAGYOZOV, L. ET AL.: "Construction of random small-insert genomic libraries highly enriched for simple sequence repeats" see (GT)15	1,4-6,8, 9
X	EUROPEAN JOURNAL OF BIOCHEMISTRY, (01 MAR 1993) VOL. 212, NO. 2, PP. 395-401., XP000673317 XODO, L. ET AL.: "SEQUENCE-SPECIFIC DNA-TRIPLEX FORMATION AT IMPERFECT HOMOPURINE-HOMOPYRIMIDINE SEQUENCES WITHIN A DNA PLASMID" see in figure 1, Rg-ap, Rt-ap, and Rg-p	1,4-6,8, 9
X	NUCLEIC ACIDS RESEARCH, vol. 16, 1988, OXFORD GB, pages 3525-3543, XP002030699 MAG, M. & ENGELS, J.: "Synthesis and structure assignments of amide protected nucleosides and their use as phosphoramidites in deoxyoligonucleotide synthesis" see table 3	1,4-8
A	FEBS LETTERS, vol. 327, August 1993, AMSTERDAM NL, pages 271-274, XP002030700 VLASSOV, V. ET AL.: "Penetration of oligonucleotides into mouse organism through mucosa and skin" cited in the application	
P,X	WO 96 24380 A (ICN PHARMACEUTICALS) 15 August 1996 see page 15, line 6 - page 24, line 6 see page 26, table 1, oligos RTC04 and RTC05 see page 27, oligos RT28, RT29, and RT30 see page 45, oligos RT18S, RT19S, RTC07S, and RTC08S	1-4,8,9, 11,16-20
1 4 X,P	EP 0 713 705 A (AKIRA KAJI) 29 May 1996 see the whole document	1,4,8,9, 11,16-20

-/--

INTERNATIONAL SEARCH REPORT

mational Application No
PCT/EP 96/05388

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 11010 A (PHARMAGENICS INC) 18 April 1996 see page 7, line 30 - page 10, line 12 see page 13, paragraph 2 see SEQ IDs 19 and 21 -----	1-4,8,9, 11,16-20

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 96/05388

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claims searched incompletely: 1-9, 11-20

* see continuation-sheet: "The scope of Formula (I) in claim 1 ..." *

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

20 inventions * see continuation-sheet *

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 96/ 05388

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

THE APPLICATION CONTAINS THE FOLLOWING INVENTIONS:

- 1) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 2, modifed forms thereof, pharmaceutical compositions containing it.

- 2) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 3, modifed forms thereof, pharmaceutical compositions containing it.

- 3) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 4, modifed forms thereof, pharmaceutical compositions containing it.

- 4) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 5, modifed forms thereof, pharmaceutical compositions containing it.

- 5) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 6, modifed forms thereof, pharmaceutical compositions containing it.

- 6) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 7, modifed forms thereof, pharmaceutical compositions containing it.

.../...

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 96/ 05388

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

-.7) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 8, modifed forms thereof, pharmaceutical compositions containing it.

- 8) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 9, modifed forms thereof, pharmaceutical compositions containing it.

- 9) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 10, modifed forms thereof, pharmaceutical compositions containing it.

- 10) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 12, modifed forms thereof, pharmaceutical compositions containing it.

- 11) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 13, modifed forms thereof, pharmaceutical compositions containing it.

- 12) Claims 1-6,8,10-20 (all partially)

An oligonucleotide defined by SEQ ID 14, modifed forms thereof, pharmaceutical compositions containing it.

- 13) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 15, modifed forms thereof, pharmaceutical compositions containing it.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

- 14) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 16, modifed forms thereof, pharmaceutical compositions containing it.

- 15) Claims 1-4,6,8-20 (all partially)

An oligonucleotide defined by SEQ ID 17, modifed forms thereof, pharmaceutical compositions containing it.

- 16) Claims 1-4,6,8,10-20 (all partially)

An oligonucleotide defined by SEQ ID 18, modifed forms thereof, pharmaceutical compositions containing it.

- 17) Claims 1-20 (all partially)

An oligonucleotide defined by SEQ ID 19, modifed forms thereof, pharmaceutical compositions containing it.

- 18) Claims 1-20 (all partially)

Oligonucleotides defined by Formula (I) of claim 1 and not covered by SEQ IDs 1 to 19, modifed forms thereof, pharmaceutical compositions containing them.

- 19) Claims 11-20 (all partially)

An oligonucleotide defined by SEQ ID 1 for use as a medicament.

- 20) Claims 11-20 (all partially)

An oligonucleotide defined by SEQ ID 11 for use as a medicament.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

The scope of Formula (I) in claim 1 is very broad and speculative as it encompasses compounds having a length varying from 2 to more than 33600 nucleotides. That means that the vast majority of the claimed compounds are in contradiction with the definition of an oligonucleotide which is restricted in length. Furthermore, the available experimental data actually only comprise a very small part of the possible oligonucleotides claimed, all being of less than 100 nucleotides in length, and therefore Formula (I) cannot be considered as a permissible generalisation fairly based on experimental evidence (Art.6 PCT).
In view of the large number of oligonucleotides which are theorically encompassed by this formula, the search has been restricted for economic reasons to the oligonucleotides cited in the examples and claimed in claim 10 (Art.17(2)(a)(ii) PCT; PCT Search Guidelines PCT/GL2, Chapter III,2.1, and 4.1), and to the oligonucleotides covered by Formula (I) and found during this search.

INTERNATIONAL SEARCH REPORT

Information on patent family members

rnational Application No
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